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Genome-wide transcriptome analysis of *Clavibacter michiganensis* subsp. *michiganensis* grown in xylem mimicking medium

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ABSTRACT

The interaction between *Clavibacter michiganensis* subsp. *michiganensis* with its host, the tomato plant (*Solanum lycopersicum*), is poorly understood and only few virulence factors are known. While studying of the bacteria *in planta* is time-consuming and difficult, the analysis *in vitro* would facilitate research. Therefore, a xylem mimicking medium (XMM) for *C. michiganensis* subsp. *michiganensis* was established in this study based on an apoplast medium for *Xanthomonas campestris* pv. *vesicatoria*. In contrast to the apoplast medium, XMM contains no sugars, but amino acids which serve as nitrogen and carbon source. As a result, growth in XMM induced transcriptional changes of genes encoding putative sugar, amino acid and iron uptake systems. In summary, mRNA levels of about 8% of all *C. michiganensis* subsp. *michiganensis* genes were changed when XMM-grown bacteria were compared to M9 minimal medium-grown cells. Almost no transcriptional changes of genes encoding hydrolytic enzymes were detected, leading to the idea that XMM reflects the situation in the beginning of infection and therefore allows the characterization of virulence factors in this early stage of infection. The addition of the plant wound substance acetosyringone to the XMM medium led to a change in transcript amount, including genes coding for proteins involved in protein transport, iron uptake and regulation processes.

1. Introduction

The genus *Clavibacter* belongs to the group of *Actinobacteria* which are defined as high G+C DNA Gram-positive bacteria. All members of the genus belong to a single species and are plant pathogenic, but differ significantly in their respective host spectrum. Despite the economic importance due to significant losses of the different crops infected, knowledge about virulence factors of *Clavibacter michiganensis* subspecies and host–pathogen interactions on a molecular level are rather limited. *C. michiganensis* subsp. *michiganensis*, which causes wilt and canker of tomato (*Solanum lycopersicum*) (Strider, 1969), is the best investigated subspecies in this respect. Three genes and their respective products have been studied in detail in *C. michiganensis* subsp. *michiganensis*: *pat-1*, encoding a serine protease (Dreier et al., 1995), *celA*, encoding a β-1,4-endoglucanase (Jahr et al., 2000) and *tomA*, coding for a tomatinase which degrades the antimicrobial saponin tomatin (Kaup et al., 2005). The genes *pat-1* and *celA* are located on natural plasmids of *C. michiganensis* subsp. *michiganensis* NCPPB382, pCM1 and pCM2. These are crucial for virulence and symptom development and besides *pat-1* and *celA*, three more putative serine proteases are encoded on these plasmids which may also play an important role in pathogenicity. The *tomA* gene is chromosomally encoded and located within the *chp*/*tomA* region, a putative pathogenicity island (Gartemann et al., 2008), which comprises also the serine protease-encoding genes *chpC*, *chpG*, *ppaA* and *ppaC*, further putative virulence factors of *C. michiganensis* subsp. *michiganensis* (Eichenlaub and Gartemann, 2011). The loss of this pathogenicity island as well as the loss of the natural plasmids pCM1 and pCM2 result in reduced pathogenicity and symptom development in tomato (Eichenlaub and Gartemann, 2011).

When the genome sequences of *C. michiganensis* subsp. *michiganensis* (Gartemann et al., 2008) and *C. michiganensis* subsp. *sepedonicus* (Bentley et al., 2008) were published and compared, the gene products of the *pat-1* and *celA* homologs were recognized as common virulence factors in *C. michiganensis* subsp. *sepedonicus*, with a *pat-1* homolog being present also in the genome of the closely related sugarcane pathogen *Leifsonia xyli* subsp. *xyli* (Monteiro-Vitorello et al., 2004).

The publication of *C. michiganensis* subsp. *michiganensis* genome sequences has not only promoted bioinformatic analyses, but allowed global analysis approaches as well and was for example the basis for proteome and transcriptome analyses published recently (Flügel et al., 2012; Savidor et al., 2012). In these studies,

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C. michiganensis subsp. *michiganensis* was grown *in vivo* or *in vitro* in a minimal medium with 5% tomato homogenate or 10% xylem sap. The addition of tomato homogenate led to a decrease transcript amount of genes coding for virulence factors like serine proteases or extracellular enzymes. Interpretation of this observation is difficult, since the macerated plant material is not well defined. The addition of xylem sap would display a good alternative to the analysis *in vivo*, but requires high amounts of xylem sap and therefore a high amount of plants. In this study, we applied whole-genome transcriptome analyses by DNA microarrays on *C. michiganensis* subsp. *michiganensis* to establish a xylem mimicking *in vitro* medium. This medium provides a time- and cost-effective basis for the analysis of interaction of *C. michiganensis* subsp. *michiganensis* and other pathogens with their host plant tomato and can be easily adapted to the composition of the xylem sap in other plants.

2. Materials and methods

2.1. Strains and growth conditions

In this study, the tomato-pathogenic *C. michiganensis* subsp. *michiganensis* wild type strain NCPPB382 was used which contains the two natural plasmids pCM1 and pCM2 (Gartemann et al., 2008). *C. michiganensis* subsp. *michiganensis* was grown at 26–28 °C in TBY medium or on TBY agar for overnight culture. For growth experiments, the bacteria were incubated at the same temperature in M9 minimal medium (0.5% glucose, 15 g/l $Na_2HPO_4 \times 12H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 2 mM methionine, 1 ml/l 0.1 M $CaCl_2$, 2 ml/l 1 M $MgSO_4$, 2.5 μl/l thiamine (200 mg/ml), 27.6 μl/l nicotinic acid (18 mg/ml), 0.2 mg/l $FeCl_3 \times 6H_2O$, 0.01 mg/l $CuCl_2 \times 2H_2O$, 0.01 mg/l $Na_2B_4O_7 \times 10H_2O$, 0.01 mg/l $MnCl_2 \times 2H_2O$, 0.01 mg/l $(NH_4)_6Mo_7 \times 4H_20$, 0.04 mg/l $ZnCl_2$) (Chalupowicz et al., 2010), *Xanthomonas* apoplast medium XVM2 (1.168 g/l NaCl, 1.32 g/l $(NH_4)_2SO_4$, 1.233 g/l $MgSO_4$, 0.147 g/l $CaCl_2$, 0.022 g/l KH_2PO_4, 0.056 g/l K_2HPO_4, 0.01 mM $FeSO_4$, 10 mM fructose, 10 mM sucrose, 0.03% (w/v) casamino acids) (Wengelnik et al., 1996) and xylem mimicking medium XMM (1.168 g/l NaCl, 2.02 g/l KNO_3, 1.233 g/l $MgSO_4$, 0.147 g/l $CaCl_2$, 0.022 g/l KH_2PO_4, 0.056 g/l K_2HPO_4, 0.01 mM $FeSO_4$, 0.2% (w/v) casamino acids). For RNA isolation, the bacteria were grown overnight in TBY medium and subsequently used to inoculate M9 and XMM medium as well as XMM medium with 20 μM acetosyringone (added at inoculation time to the medium). The cells were harvested at optical densities between 0.35 and 0.55 (see Fig. 1).

2.2. Preparation of xylem sap

Tomato xylem sap was collected from four weeks old tomato plants (*S. lycopersicum* cv. Moneymaker) The stem was cut about 10 cm above the soil and the xylem sap was collected after 5 min to avoid contamination with phloem sap. Collection of xylem sap was carried out for about half an hour and the sap of all plants (15–20 plants) was pooled. Afterwards, the sap was sterile-filtrated (0.2 μm pore size) and frozen at −20 °C (Rellan-Alvarez et al., 2011). Xylem sap collection was carried out twice.

2.3. Ammonium and nitrate measurements

To determine NH_4^+ concentrations, a modified indophenol test according to Jahns et al. (1988) was used. Specific sample volume was filled to 1 ml with 50 mM KH_2PO_4 buffer, pH 7. After 10 min at 37 °C, 1 ml of solution I (4.72% (v/v) phenol, 0.025% (w/v) sodium nitroprusside) and 1 ml of solution II (2.5% (w/v) sodium hydroxide, 5.245% (v/v) sodium hypochlorite) was added. Subsequently, the samples were heated at 50 °C for 15 min and absorption was measured at 546 nm. A calibration curve was obtained with different concentrations of NH_4Cl. For the determination of nitrate concentration, the nitrate determination kit (Roche, Penzberg) was used, according to the manufacturer description. For ammonium and nitrate measurements, two to three biological and two technical replicates were carried out.

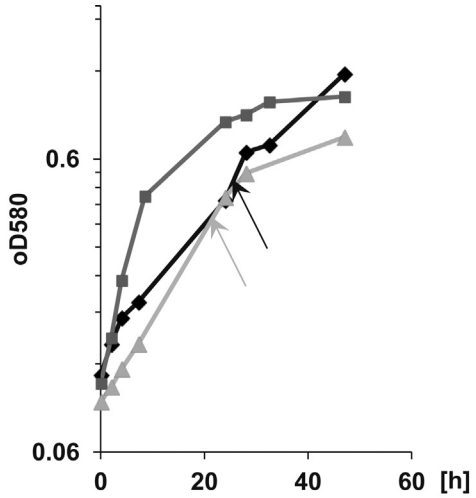

Fig. 1. Growth of *Cmm*382 in different media. *Cmm*382, grown in TBY medium was used to inoculate minimal media with an initial oD_{580} of 0.1. Growth in minimal medium (M9, black), *Xanthomonas* apoplast medium (XVM2, dark grey) and xylem mimicking medium (XMM, light grey) was analyzed; experiments were carried out independently twice or thrice and typical results are shown. For RNA isolation, bacteria were harvested at an oD_{580} between 0.4 and 0.55 after growth in M9 medium (black arrow) and between 0.35 and 0.45 after growth in XMM medium (light grey arrow).

2.4. Sugar and amino acids determination

For extraction of amino acids and sugars, five volumes of ethanol were added to 100 μl of the xylem sap, followed by incubation at 80 °C for 60 min. After cooling for 15 min and centrifugation for 5 min at 14,500 × *g*, supernatants were dried for 60–90 min in a Savant DNA SpeedVac concentrator (Thermo Scientific, Langenselbold) and resuspended in 250 μl of water. For determination of amino acid concentrations, 10 μl of the extracts were derivatized, using the fluorophore 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Cohen and Michaud, 1993). 10 μl of this solution were subsequently analyzed using a Summit reversed phase HPLC system (Dionex, Idstein) with a fluorescence detector RF2000 (Dionex, Idstein) as described (Hofmann et al., 2011). In brief, for separation, a Luna C18(2) column (5 μm, 250 mm × 4.6 mm) with a security guard precolumn (4.0 mm × 3.0 mm) (Phenomenex, Aschaffenburg) was used. Amino acids were eluted as described with a trimeric gradient of buffer A (140 mM sodium acetate, 7 mM triethanolamin, pH 6.2), eluent B (acetonitril) and eluent C (H_2O_{bidest}) within a total run time of 60 min at a flow rate of 1 ml/min and a column temperature of 37 °C (Hofmann et al., 2011). The contents of amino acids were calculated based on peak areas relative to pure standards in a range of 4–200 nmol. For the concentration measurement of sugars, glucose, fructose and sucrose were quantified enzymatically from ethanolic extracts using a coupled optical test at 340 nm as described (Stitt et al., 1989) in a total assay volume of 200 μl using a microtiter plate reader (BioTek, Bad Friedrichshall). The determination of sugar and amino acid concentration was carried out in two biological and two technical replicates.

2.5. RNA preparation

C. michiganensis subsp. *michiganensis* RNA was prepared from 50 ml culture sample using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren). The cells were suspended in 350 μl RA1 buffer with 1% (w/v) 2-mercaptoethanol and immediately disrupted using glass beads and a homogenizer (precellys 24, PEQLAB Biotechnologie GMBH, Erlangen). Disruption was performed by two 30 s cycles at a speed of 6.5 ms^{-1}. After cell debris was removed, RNA was isolated following the supplier's recommendation. A further DNase digestion was performed with Turbo-DNase (Ambion, Austin, TX) to completely remove chromosomal DNA contaminations. RNA samples were stored at −80 °C.

2.6. Design of DNA microarrays, RNA quality control, labeling, hybridization and analysis

Whole genome expression analyses were performed using custom *C. michiganensis* subsp. *michiganensis* 8 × 15 K microarrays (Agilent, Santa Clara). Based on the 3107 annotated genes downloaded from the JCVI.CMR genome browser (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi), one specific probe (45–60mer) per gene was designed using the eArray software (https://earray.chem.agilent.com). These oligonucleotides, representing the 3107 *C. michiganensis* subsp. *michiganensis* genes, were spotted four or five times together with internal controls. Genes from genomic DNA and from the plasmids pCM1 and pCM2 were considered, as well as pseudogenes.

Total RNA from two to four biological replicates of *C. michiganensis* subsp. *michiganensis* from different conditions (M9, XMM and XMM with 20 μM acetosyringone) was isolated as described above. RNA quantity was quantified with the ND-100 spectrophotometer v3.3.0 (NanoDrop Technologies), and RNA was verified using an Agilent RNA 6000 Nano Chip on an Agilent 2100 BioAnalyzer as recommended (Agilent RNA 6000 Nano Assay Protocol2). A total of 300 ng RNA were reverse transcribed using a random T7N9 primer (Moreno-Paz and Parro, 2006). Further sample labeling and hybridization was essentially performed as described in the two-color microarray-based gene expression analysis protocol provided by Agilent (v5.7, 2008; Agilent Technologies, Santa Clara). For each replicate, the Cy5 dye correlated with the control sample and the Cy3 dye with the experimental sample. Slides were scanned on the Agilent Microarray Scanner with extended dynamic range (XDR) at high resolution (5 μm). Comparison was carried out between bacteria grown in M9 and XMM medium as well as between bacteria grown in XMM medium and XMM medium with 20 μM acetosyringone. Data sets were extracted by the feature extraction software package (v9.5.3.1/Agilent Technologies) using a standard protocol. Data were analyzed using the GeneSpring XI software (Agilent Technologies) with standard settings. Statistically significantly deregulated genes (p-value < 0.05) were identified using the *t-test against zero* function of GeneSpring XI and the Benjamini–Hochberg correlation (Benjamini and Hochberg, 1995).

3. Results and discussion

3.1. Development of an in vitro xylem medium

Since *C. michiganensis* subsp. *michiganensis in planta* growth is in the initial phase of an infection restricted to the xylem of tomato plants, it is difficult to collect high amounts of bacterial biomass from this source to study host pathogen interactions. To circumvent this problem, Flügel et al. (2012) used tomato homogenate as an additive to defined minimal medium in a recent publication. As an alternative, we developed a xylem mimicking medium based on a previously published medium used for the analysis of *Xanthomonas campestris* pv. *vesicatoria* which colonizes the apoplast of tomato plants (Wengelnik et al., 1996). The originally described apoplast medium (XVM2) contained sugars which are present in the apoplast but typically absent in the xylem. As nitrogen source, ammonium and a mixture of amino acids was added. Ammonium is toxic to many plants but is at least discussed to by present in the xylem (Husted et al., 2000), where nitrate is the typical nitrogen transport form.

As a basis for further experiments, the concentrations of key compounds in the xylem sap of four weeks old tomato plants were determined. For this purpose, the plants were cut and the xylem sap was collected from the stems for about half an hour. To avoid contamination with phloem content, xylem sap collected during the first 5 min was discarded. Subsequently, the concentrations of nitrate, ammonium, different sugars (glucose, fructose and sucrose) and amino acids were determined. Nitrate concentration reached 33.34 ± 1.37 mM, compared with an ammonium concentration of only 1.88 ± 0.16 mM, indicating that nitrate is clearly preferred as nitrogen transport compound in tomato. Glucose, fructose and sucrose were not detectable in the xylem sap, indicating that no phloem contamination had occurred. The most prominent amino acids detected were glutamine and asparagine; for all other amino acids, ten times lower concentrations were measured. As observed previously (Bialczyk et al., 2004), the concentrations of the various amino acids varied strongly (Table 1) between the two biological replicates, while their ratio stayed constant (data not shown). Based on these data, composition of the *Xanthomonas* apoplast medium (XVM2) was changed in respect to sugars and ammonium, which were omitted and an increased casamino acid content, resulting in xylem mimicking medium XMM (Table 1). The measurement of nitrate, ammonium, sugar and amino acid concentrations in XMM

Table 1
Composition of tomato plant xylem sap and xylem mimicking medium (XMM). The concentrations of cysteine and tryptophan were not determined. Two rounds of harvesting were carried out with 15–20 plants each. The xylem sap of each harvest was pooled. Mean and deviation of the two pooled preparations are given for amino acid and sugar concentrations. Nitrate and ammonium data represent mean and standard deviation of two biological and three technical replicates.

Compound	Xylem sap	Xylem mimicking medium[a]
Nitrate	33.34 ± 1.37 mM	23.4 ± 5.1 mM
Ammonium	1.88 ± 0.16 mM	1.56 ± 0.3 mM
Sugar	(mM)	(mM)
Glucose, fructose, sucrose	0	0
Amino acids	(μM)	(μM)
Gln	40–557	33–79
Asn	20–131	0
Ala	2.2–61	88–220
Pro	3.3–44	174–448
His	5.3–42	22–56
Leu	5.5–36	126–294
Val	7.6–35	93–224
Thr	5.9–33	38–114
Glu	8–30	176–414
Arg	7–27	66–164
Lys	7.8–26	66–163
Ser	7.8–26	90–219
Ille	6–24	66–141
Asp	6.4–22	71–172
Gly	2.7–11	46–118
Phe	1.5–11	52–134
Tyr	1.6–9	10–26
Met	0.3–0.4	16–40

[a] Data represent mean values and standard deviations from three independent medium preparations with different charges of casamino acid, replicates of transcriptome analyses were carried out with one batch of XMM.

medium resulted in a lower amount of nitrate (23.4 ± 5.1 mM) compared to the amount in xylem sap (33.34 ± 1.37 mM). Since nitrate cannot be assimilated by *C. michiganensis* subsp. *michiganensis*, the amount of nitrate should not be crucial. Despite the fact that no ammonium was added to XMM, a low ammonium concentration (1.56 ± 0.3 mM) was measured. Most likely this is the result of a release of ammonium due glutamine decomposition, which easily takes place due to glutamine instability at higher temperatures and was already found previously (Nolden, Burkovski; unpublished). A similar low ammonium concentration was observed in xylem sap (1.88 ± 0.16 mM). In this case, however, the source of ammonium, cannot be determined unequivocally; however since ammonium is toxic for many plants, decomposition of amino acids during the ammonium measurement is most likely and ammonium was consequently omitted from XMM. Asparagine, representing one of the major amino acids in xylem sap, is lacking in XMM medium, since it is almost absent in casamino acids, which were used as source of amino acids. Based on the fact that glutamate, proline and arginine can be used as carbon and nitrogen source by *C. michiganensis* subsp. *michiganensis* (Romano and Nickerson, 1958), the species is not asparagine-auxotrophic and no growth defect was observed, no extra asparagine was added.

For a basic characterization, growth tests were carried out (Fig. 1). Beside XMM, M9 medium was used as an established minimal medium for *C. michiganensis* subsp. *michiganensis* and XVM2 as basic medium. Growth rates obtained were 11.2 h for XMM, 12.1 h for M9 and 3.97 h for XVM2 (Fig. 1). Compared to M9 and XMM, the faster growth of *C. michiganensis* subsp. *michiganensis* in XVM2 reflects the rich composition of this medium. Since growth of the bacteria was reported to be slow in tomato plants (Savidor et al., 2012), the conditions in XMM seem to better reflect the conditions in the xylem sap.

3.2. Transcriptome analyses by DNA microarrays

For *C. michiganensis* subsp. *michiganensis*, only few virulence factors are characterized (Pat-1, CelA) or suspected (ChpC, ChpG, PpaA, PpaC). For the analysis of further virulence factors, gene expression of *C. michiganensis* subsp. *michiganensis* grown in xylem mimicking medium was compared with transcription in minimal medium, using DNA microarray experiments (Table 2). Out of the 3107 *C. michiganensis* subsp. *michiganensis* genes represented on the microarrays, the transcript level of 179 genes was higher in minimal medium compared with xylem mimicking medium. These genes were classified according to functional categories (COG group, Table 2 and Fig. 2) as described by Flügel et al. (2012). 42 of these genes (23%) belong to the fourth COG group (potentially relevant in the phytopathogenic interaction), amongst them 47% regulator-encoding genes were found (Fig. 2 and Supplementary Table 2). The gene product of one of these genes, CMM_2645, was also identified in proteome analysis after growth of the bacteria in minimal medium (Savidor et al., 2012). The transcript amount of CMM_1624 was also found to be lower in minimal medium with 5% tomato homogenate compared to minimal medium (Flügel et al., 2012). The gene which showed the highest change in transcript amount code for an iron-siderophore ABC transporter (CMM_2931 (*fecB2*): 6.8), leading to the suggestion that the access to iron in XMM medium is not limited and does not require siderophores. This is consistent with the results of the transcriptome analysis after growth of *C. michiganensis* subsp. *michiganensis* in minimal medium with 5% tomato homogenate (Flügel et al., 2012).

The transcript level of 247 genes was higher in xylem mimicking medium compared to minimal medium (Table 2 and Fig. 3). The gene with the highest change in transcript amount codes for the phosphoenolpyruvate carboxykinase (CMM_1473 (*pckA*), Table 3). 94 genes (38%) belong to the fourth *C. michiganensis* subsp. *michiganensis* COG group, amongst them 75% which code for transporters, mainly sugar transporter components (32 genes) but also amino acid transporter components (five genes) (Fig. 3 and Supplementary Table 1). Despite the fact that the XMM medium

Table 2
Overview of genes which showed altered transcript amounts in microarray experiments with RNA from *C. michiganensis* subsp. *michiganensis* strain *Cmm*382 grown in M9 and XMM.

COG	Category	Genes	Higher mRNA level in	
			M9	XMM
I	Metabolism	97	42	55
I.1	Energy and carbon metabolism	32	6	29
I.2	Amino acid metabolism	30	17	12
I.3	Nucleotide metabolism	6	5	2
I.4	Lipid metabolism	9	1	8
I.5	Metabolism of cofactors and vitamins	13	7	4
I.6	Secondary metabolism	11	6	0
II	Cellular processes	12	7	5
II.1	Cell cycle	0	1	0
II.2	Cell wall	11	6	5
III	Information storage and processing	32	29	3
III.1	Replication and repair	12	12	1
III.2	Transcription	2	3	0
III.3	Translation	14	14	2
IV	Potentially relevant in the phytopathogenic interaction	134	42	92
IV.1	Transporters	82	13	68
IV.2	Protein transport	3	3	1
IV.3	Regulators	33	18	10
IV.4	Stress	5	5	2
IV.5	Resistance	11	2	6
IV.6	Extracellular enzymes	4	1	3
IV.7	Intracellular proteases	2	0	2
V	Poorly characterized	151	59	92
V.1	General function prediction only	30	9	22
V.2	Function unknown	115	50	70
Sum		426	179	247

Table 3
Changed transcript amount of *Cmm*382 genes of COG IV (potentially relevant in phytopathogenic interaction) in xylem mimicking medium compared to standard minimal medium. Selected genes are listed here. Genes which showed a decreased transcript amount in XMM medium are listed with a negative FCA. The whole list can be found in the Supplementary material, Supplementary Tables 1 and 2. FCA: fold change absolute, VI.1: transporters, VI.5: resistance, VI.6: extracellular enzymes.

GenDB-ID	Possible function	COG	FCA
CMM_1473	Phosphoenolpyruvate carboxykinase	I.1	34.4
CMM_0196	Sugar ABC transporter, substrate-binding protein	VI.1	30.9
CMM_0197	Sugar ABC transporter, permease component	VI.1	23.8
CMM_2783	Sugar ABC transporter, substrate-binding protein	VI.1	20.0
CMM_1314	Iron ABC transporter, substrate-binding protein	VI.1	10.9
CMM_0795	Extracellular nuclease/phosphatase	VI.6	5.5
CMM_0094	*cytA*, cytochrome P450	VI.5	4.7
CMM_0095	*cytB*, 3Fe-4S ferredoxin	VI.5	4.9
CMM_0096	*cytC*, ferredoxin reductase	VI.5	2.3
CMM_1674	*xysB*, endo-1,4-β-xylanase B	VI.6	3.9
CMM_2535	*sbtB*, serine protease, peptidase family S8A	VI.6	2.6
CMM_2931	Iron-siderophore ABC transporter, substrate-binding protein	VI.1	−6.8
CMM_1129	Siderophore-interacting protein	I.6	−3.9

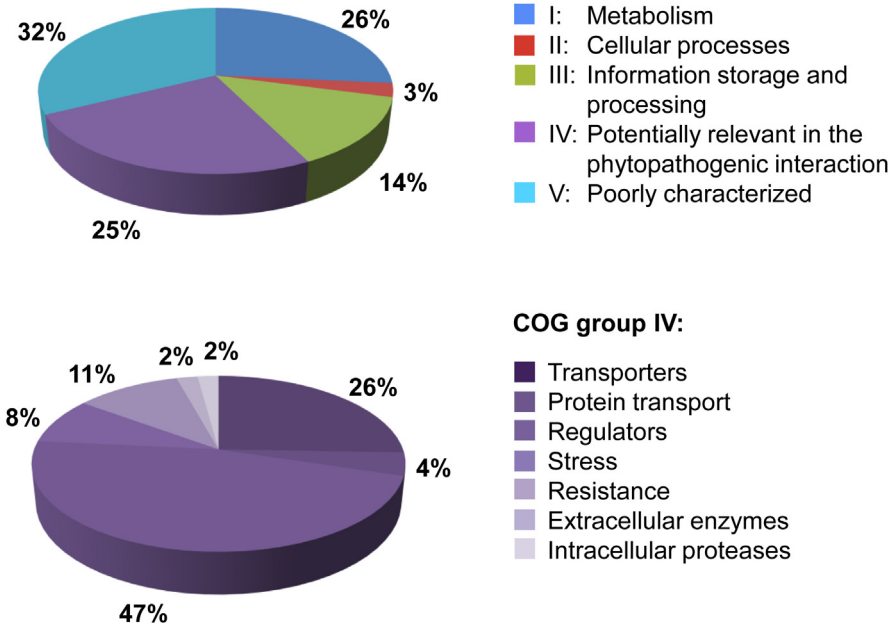

Fig. 2. Functional categories of *C. michiganensis* subsp. *michiganensis* genes that showed a higher transcript amount in minimal medium M9 compared with xylem mimicking medium XMM. DNA microarray analyses were carried out with RNA isolated from wild type cells grown in minimal medium and xylem surrogate medium. The 179 genes with decreased transcript levels were classified according to functional categories (Flügel et al., 2012).

contains no sugars, the transcript amounts of many genes coding for sugar transporters were higher in this medium compared to M9 medium. The most likely explanation for this observation might be a repression of expression of these transporters by the presence of glucose in M9 due to a carbon catabolite repression mechanism. According to the annotation of the different genes, transport of proline (CMM_1532), glutamate (CMM_2006, CMM_2007) and isoleucine/leucin/valine (CMM_2562) is facilitated in XMM and in fact, these are major amino acids found in this medium.

The genome of *C. michiganensis* subsp. *michiganensis* NCPPB382 contains three genes which code for iron transporters. The transcript amount of all of these genes was higher after growth in XMM medium compared to the amount after growth in M9 medium. These findings led to the first assumption that XMM mimics the condition in the xylem at an early stage of infection when nutrient availability is poor and high substrate affinity ABC transporters are crucial for a sufficient nutrient supply. Two of the transporters were antimicrobial peptide transporters (genes: CMM_0684 and CMM_0685) which act both as sensors for antimicrobial peptides and as detoxification systems in *Bacillus subtilis* (Kallenberg et al., 2013). Beside transporter-encoding genes, 12% of the genes in COG group IV code for regulators which might play an important role in the regulation of virulence factors. 7% were resistance genes, amongst them the *cytABC*-operon, whose gene products

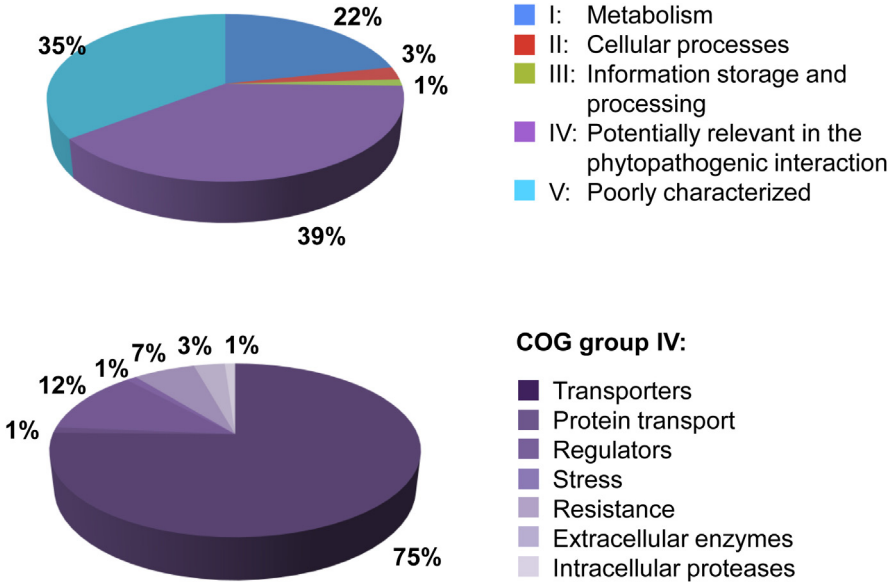

Fig. 3. Functional categories of *C. michiganensis* subsp. *michiganensis* genes that showed a higher transcript amount in xylem surrogate medium XVMN compared with minimal medium M9. DNA microarray analyses were carried out with RNA isolated from wild type cells grown in minimal medium and xylem surrogate medium. The 247 genes with increased transcript levels were classified according to functional categories (Flügel et al., 2012).

Table 4
Overview of genes which showed altered transcript amount, identified in microarray experiments with RNA from *C. michiganensis* subsp. *michiganensis* strain *Cmm*382 grown in XMM and XMM + acetosyringone (AS).

COG	Category	Genes	Higher mRNA level in	
			XMM	XMM + AS
I	Metabolism	85	31	54
II	Cellular processes	31	18	13
III	Information storage and processing	27	12	15
IV	Potentially relevant in the phytopathogenic interaction	155	65	90
IV.1	Transporters	81	28	53
IV.3	Regulators	37	15	22
IV.6	Extracellular enzymes	3	2	1
V	Poorly characterized	170	72	98
Sum		468	198	270

can degrade cyclic amines like morpholines in *Mycobacterium* sp. strain HE5 (Schräder et al., 2000; Sielaff and Andreesen, 2005), and a glutaredoxin (CMM_2111) which can act as an antioxidant (Berndt et al., 2007). 3% of the genes in the COG group IV were encoding extracellular enzymes (CMM_0795: putative extracellular nuclease phosphatase, CMM_1674: *xysB*, endo-1,4-β-xylanase B, CMM_2535: *sbtB*, putative serine protease, peptidase family S8A) (Table 3 and Supplementary Table 1). The increased *xysB* mRNA level, also observed *in planta* by Chalupowicz et al. (2010) might be connected to xylanase activity, which was reported for *C. michiganensis* subsp. *michiganensis* previously (Meletzus et al., 1993). That xylanases could be important for host–pathogen interaction was shown in the phytopathogen fungus *Botrytis cinerea*, where the transcript amount of a xylanase was increased in early stage of infection (12 h post infection) (Brutus et al., 2005), and in the fungus *Fusarium graminearum*, where a xylanase induced plant cell death and hydrogen peroxide accumulation (Sella et al., 2013).

Almost no transcriptional changes of genes encoding hydrolytic enzymes were detected, supporting the idea, that XMM reflects the situation in the beginning of infection. At this early stage of infection, the main goals of *C. michiganensis* subsp. *michiganensis* seem to be nutrient uptake with different ABC transporters (sugar, amino acid and iron), multiplication and spreading in the xylem vessels. Different prerequisites could be important for the expression and secretion of hydrolytic enzymes. Examples are a high bacterial density, degradation products or plant signal substances. These plant signal substances could be phenolic substances like acetosyringone, a wound-induced compound which display a signal for a substance-specific expression change of virulence factors of *Agrobacterium tumefaciens* (Stachel et al., 1985).

In fact, the addition of 20 μM acetosyringone, which did not influence growth (data not shown), led to a change in *C. michiganensis* subsp. *michiganensis* transcription patterns (Table 4 and Supplementary Tables 3 and 4), however in an unexpected magnitude. The transcript level of 198 genes was decreased and of 270 genes was increased in XMM medium with 20 μM acetosyringone compared with XMM medium. About 33% of these latter genes could be dedicated to COG group IV. The transcript amounts of three genes which encode iron siderophore transporter components were more than two fold higher (CMM_0166/7 (*fhuB/D*) and CMM_2931 (*fecB2*)). This may indicate, that *C. michiganensis* subsp. *michiganensis* recognizes acetosyringone as a signal for an altered iron supply. Six genes coding for proteins which are involved in protein transport showed higher transcript amounts after growth in XMM medium, beneath them genes which encode a signal peptidase (CMM_0147) and pilus proteins (CMM_1247, CMM_0924 and CMM_1301). As it is the case in some Gram-negative (Mhedbi-Hajri et al., 2011) and Gram-positive bacteria (Mandlik et al., 2008), pili might play a role in adhesion of *C. michiganensis* subsp. *michiganensis* to the host cell and could therefore be important for the host–pathogen interaction.

Also several genes of COG group II belonging to different EPS gene clusters, were changed. Eight out of 13 genes of the EPS IV cluster, encoding the major EPS synthesis pathway, showed decreased expression, while four genes from clusters EPS I, EPS II and EPS III revealed increased mRNA levels upon acetosyringone addition.

4. Conclusion

In planta experiments reflect real life of a bacterial plant pathogen and are the gold standard to study host pathogen interactions. However, they have the disadvantage of being technically demanding as well as time, space and cost intensive. Consequently, *in vitro* experiments are often useful, especially for screening purposes.

Recently, Flügel et al. (2012) reported the use of a minimal medium with 5% tomato homogenate which seems to be especially useful to characterize putative virulence gene expression in a late stage of infection. In this study, the generation and characterization of a medium mimicking the xylem content, and consequently, the early stage of infection, is reported. In contrast to the basic apoplast medium XVM2, in which virulence factors of *Xanthomonas* showed a change in gene expression, the XMM medium is containing amino acids instead of sugars which can be used by *C. michiganensis* subsp. *michiganensis* as carbon, energy and nitrogen source. Nitrate added instead of ammonium to the medium, and being a major nitrogen transport compound in the xylem sap, cannot be assimilated by *C. michiganensis* subsp. *michiganensis* as deduced from growth experiments (data not shown). Since nitrate is available in the xylem sap, it could present a signal substance for a change in bacterial gene expression as it is the case in the Gram-negative bacterium *Pseudomonas aeruginosa* (Filiatrault et al., 2005). Bacteria growing in this medium are adapting to a poor nutrient environment by expression of various nutrient uptake systems. Expression of already known virulence factors was, as in the study of Flügel et al. (2012), not a major response. The latter observation might be the result of an induction of virulence genes taking place already in minimal medium used as comparison for *in vitro* or *in planta* conditions or of time-dependent expression patterns making the optimal time point for sampling critical (Chalupowicz et al., 2010; Flügel et al., 2012).

In summary, XMM seems to mimic the conditions in the xylem at an early time point of infection and therefore allows the characterization of virulence factors which are important in an early stage of host–pathogen interaction. Furthermore, the medium allows the testing of putative signal substances, as shown for the plant wound substance acetosyringone in this study.

Authors' contributions

EH carried out growth and fluorescence measurements, determination of NH_4^+ and NO_3^- concentration, RNA isolation and analysis of the microarray data. SA contributed to the setup of XMM. Microarray experiments were carried out by SR and SS helped with the analysis of microarray data. JH determined sugar and amino acid concentrations. AB supervised the experiments of EH and SA and was responsible for the draft and final version of the manuscript. All authors approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2013.09.006.

References

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B: Stat. Methodol. 57, 289–300.

Bentley, S.D., Corton, C., Brown, S.E., Barron, A., Clark, L., Doggett, J., Harris, B., Ormond, D., Quail, M.A., May, G., Francis, D., Knudson, D., Parkhill, J., Ishimaru, C.A., 2008. Genome of the actinomycete plant pathogen *Clavibacter michiganensis* subsp. *sepedonicus* suggests recent niche adaptation. J. Bacteriol. 190, 2150–2160.

Berndt, C., Lillig, C.H., Holmgren, A., 2007. Thiol-based mechanisms of the thioredoxin and glutaredoxin systems: implications for diseases in the cardiovascular system. Am. J. Physiol. Heart Circ. Physiol. 292, H1227–H1236.

Bialczyk, J., Lechowski, Z., Dziga, D., 2004. Composition of the xylem sap of tomato seedlings cultivated on media with HCO_3^- and nitrogen source as NO_3^- or NH_4^+. Plant Soil 263, 265–272.

Brutus, A., Reca, I.B., Herga, S., Mattei, B., Puigserver, A., Chaix, J.C., Juge, N., Bellincampi, D., Giardina, T., 2005. A family 11 xylanase from the pathogen *Botrytis cinerea* is inhibited by plant endoxylanase inhibitors XIP-I and TAXI-I. Biochem. Biophys. Res. Commun. 337, 160–166.

Chalupowicz, L., Cohen-Kandli, M., Dror, O., Eichenlaub, R., Gartemann, K.H., Sessa, G., Barash, I., Manulis-Sasson, S., 2010. Sequential expression of bacterial virulence and plant defense genes during infection of tomato with *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 100, 252–261.

Cohen, S.A., Michaud, D.P., 1993. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. Anal. Biochem. 211, 279–287.

Dreier, J., Bermpohl, A., Eichenlaub, R., 1995. Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 85, 462–468.

Eichenlaub, R., Gartemann, K.H., 2011. The *Clavibacter michiganensis* subspecies: molecular investigation of Gram-positive bacterial plant pathogens. Annu. Rev. Phytopathol. 49, 445–464.

Filiatrault, M.J., Wagner, V.E., Bushnell, D., Haidaris, C.G., Iglewski, B.H., Passador, L., 2005. Effect of anaerobiosis and nitrate on gene expression in *Pseuomonas aeruginosa*. Infect. Immun. 73, 3764–3772.

Flügel, M., Becker, A., Gartemann, K.H., Eichenlaub, R., 2012. Analysis of the interaction of *Clavibacter michiganensis* subsp. *michiganensis* with its host plant tomato by genome-wide expression profiling. J. Biotechnol. 160, 42–54.

Gartemann, K.H., Abt, B., Bekel, T., Burger, A., Engemann, J., Flügel, M., Gaigalat, L., Goesmann, A., Gräfen, I., Kalinowski, J., Kaup, O., Kirchner, O., Krause, L., Linke, B., McHardy, A., Meyer, F., Pohle, S., Rückert, C., Schneiker, S., Zellermann, E.M., Pühler, A., Eichenlaub, R., Kaiser, O., Bartels, D., 2008. The genome sequence of the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 reveals a large island involved in pathogenicity. J. Bacteriol. 190, 2138–2149.

Hofmann, J., Börnke, F., Schmiedl, A., Kleine, T., Sonnewald, U., 2011. Detecting functional groups of *Arabidopsis* mutants by metabolic profiling and evaluation of pleiotropic responses. Front. Plant Sci. 2, 82.

Husted, S., Hebbern, C.A., Mattsson, M., Schjoerring, J.K., 2000. A critical experimental evaluation of methods for determination of NH_4^+ in plant tissue, xylem sap and apoplastic fluid. Physiol. Plant. 109, 167–179.

Jahns, T., Zobel, A., Kleiner, D., Kaltwasser, H., 1988. Evidence for carrier-mediated, energy-dependent uptake of urea in some bacteria. Arch. Microbiol. 149, 377–383.

Jahr, H., Dreier, J., Meletzus, D., Bahro, R., Eichenlaub, R., 2000. The endo-β-1,4-glucanase CelA of *Clavibacter michiganensis* subsp. *michiganensis* is a pathogenicity determinant required for induction of bacterial wilt of tomato. Mol. Plant Microbe Interact. 13, 703–714.

Kallenberg, F., Dintner, S., Schmitz, R., Gebhard, S., 2013. Identification of important regions for resistance and signalling within the antimicrobial peptide transporter BceAB of *Bacillus subtilis*. J. Bacteriol. [Epub ahead of print].

Kaup, O., Gräfen, I., Zellermann, E.M., Eichenlaub, R., Gartemann, K.H., 2005. Identification of a tomatinase in the tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. Mol. Plant Microbe Interact. 18, 1090–1098.

Mandlik, A., Swierczynski, A., Das, A., Ton-That, H., 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends Microbiol. 16, 33–40.

Meletzus, D., Bermpohl, A., Dreier, J., Eichenlaub, R., 1993. Evidence for plasmid-encoded virulence factors in the phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. J. Bacteriol. 175, 2131–2136.

Mhedbi-Hajri, N., Jacques, M.A., Koebnik, R., 2011. Adhesion mechanisms of plant-pathogenic *Xanthomonadaceae*. Adv. Exp. Med. Biol. 715, 71–89.

Monteiro-Vitorello, C.B., Camargo, L.E.A., Van Sluys, M.A., Kitajima, J.P., Truffi, D., do Amaral, A.M., Harakava, R., de Oliveira, J.C.F., Wood, D., de Oliveira, M.C., Miyaki, C., Takita, M.A., da Silva, A.C.R., Furlan, L.R., Carraro, D.M., Camarotte, G., Almeida, N.F., Carrer, H., Coutinho, L.L., El-Dorry, H.A., Ferro, M.I.T., Gagliardi, P.R., Giglioti, E., Goldman, M.H.S., Goldman, G.H., Kimura, E.T., Ferro, E.S., Kuramae, E.E., Lemos, E.G.M., Lemos, M.V.F., Mauro, S.M.Z., Machado, M.A., Marino, C.L., Menck, C.F., Nunes, L.R., Oliveira, R.C., Pereira, G.G., Siqueira, W., de Souza, A.A., Tsai, S.M., Zanca, A.S., Simpson, A.J.G., Brumbley, S.M., Setúbal, J.C., 2004. The genome sequence of the Gram-positive sugarcane pathogen *Leifsonia xyli* subsp. *xyli*. Mol. Plant Microbe Interact. 17, 827–836.

Moreno-Paz, M., Parro, V., 2006. Amplification of low quantity bacterial RNA for microarray studies: time-course analysis of *Leptospirillum ferrooxidans* under nitrogen-fixing conditions. Environ. Microbiol. 8, 1064–1073.

Rellan-Alvarez, R., Lopez-Gomollon, S., Abadia, J., Alvarez-Fernandez, A., 2011. Development of a new high-performance liquid chromatography-electrospray ionization time-of-flight mass spectrometry method for the determination of low molecular mass organic acids in plant tissue extracts. J. Agric. Food Chem. 59, 6864–6870.

Romano, A.H., Nickerson, W.J., 1958. Utilization of amino acids as carbon source by *Streptomyces fradiae*. J. Bacteriol. 75, 161–166.

Savidor, A., Teper, D., Gartemann, K.H., Eichenlaub, R., Chalupowicz, L., Manulis-Sasson, S., Barash, I., Tews, H., Mayer, K., Giannone, R.J., Hettich, R.L., Sessa, G., 2012. The *Clavibacter michiganensis* subsp. *michiganensis*-tomato interactome reveals the perception of pathogen by the host and suggests mechanisms of infection. J. Proteome Res. 11, 736–750.

Schräder, T., Schuffenhauer, G., Sielaff, B., Andreesen, J.R., 2000. High morpholine degradation rates and formation of cytochrome P450 during growth on different cyclic amines by newly isolated *Mycobacterium* sp. strain HE5. Microbiology 146, 1091–1098.

Sella, L., Gazzetti, K., Faoro, F., Odorizzi, S., D'Ovidio, R., Schäfer, W., Favaron, F., 2013. A *Fusarium graminearum* xylanase expressed during wheat infection is a necrotizing factor but is not essential for virulence. Plant Physiol. Biochem. 64, 1–10.

Sielaff, B., Andreesen, J.R., 2005. Analysis of the nearly identical morpholine monooxygenase-encoding *mor* genes from different *Mycobacterium* strains and characterization of the specific NADH:ferredoxin oxidoreductase of this cytochrome P450 system. Microbiology 151, 2593–2603.

Stachel, S.E., Messens, E., Vanmontagu, M., Zambryski, P., 1985. Identification of the signal molecules produced by wounded plant-cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318, 624–629.

Stitt, M., Lilley, R.M., Gerhardt, R., Heldt, H.W., 1989. Metabolite levels in specific cells and subcellular compartments of plant-leaves. Method Enzymol. 174, 518–552.

Strider, D.L., 1969. Bacterial canker of tomato caused by *Corynebacterium michiganense*. A literature review and bibliography. North Carolina Agricultural Experimental Station. Tech. Bull., 193.

Wengelnik, K., Marie, C., Russel, M., Bonas, U., 1996. Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv. *vesicatoria* essential for pathogenicity and induction of the hypersensitive reaction. J. Bacteriol. 178, 1061–1069.